Low Genetic Diversity of Oval Squid, *Sepioteuthis* cf. *lessoniana* (Cephalopoda: Loliginidae), in Japanese Waters Inferred from a Mitochondrial DNA Non-coding Region[1]

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Abstract: Genetic diversity and population structure of Japanese populations of the oval squid, *Sepioteuthis* cf. *lessoniana*, were compared with populations from Taiwan and Vietnam using nucleotide sequences of the mitochondrial DNA non-coding region 2. In total, 402 nucleotide sequences representing 242 individuals from Japanese waters (Ishikawa, Japan Sea coast of Honshu; Tokushima, eastern Shikoku; Nagasaki, western Kyushu; and Okinawajima and Ishigakijima Island, in the Ryukyu Archipelago) and the East and South China Seas (Keelung, northern Taiwan; Vietnam, Gulf of Tonkin) were examined. Among the 29 haplotypes recognized, haplotype no. 1 was shared by more than 75% of individuals from Japanese localities, whereas it was found in less than 13% of specimens from the East and South China Seas populations. Conversely, the East and South China Seas populations included more than 30% individuals with haplotype no. 2, whereas less than 10% of haplotype no. 2 individuals were from Japanese localities. The differences of haplotype and nucleotide diversities between pooled Japanese populations (0.2639, 0.23%) and the East and South China Seas populations (0.7900, 1.01%) indicate that *S.* cf. *lessoniana* from Japanese waters exhibits lower genetic diversity. An analysis of molecular variance between the Japanese populations and the East and South China Seas populations was highly significant. A minimum spanning tree of 29 haplotypes and an Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree based on pairwise F_{ST} comparisons also supported the separation between Japanese and the East and South China Seas populations. We suggest that the Kuroshio Current physically limits gene flow and has thus caused the differences in genetic diversity among the populations examined.

Oval squid, *Sepioteuthis lessoniana* Férussac, 1831, in Lesson (1830–1831), is widely distributed in the Indo-Pacific area and has been regarded as a valuable fishery resource (Roper et al. 1984). However, it was reported that *S. lessoniana* may consist of a species complex because of different reproductive features around Ishigakijima Island in Japan (Segawa et al. 1993*a*,*b*). In addition, previous studies using allozyme analysis have sug-

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gested that in Japan, so-called "*S. lessoniana*" includes at least three biological species (Izuka et al. 1994, 1996). Izuka et al. (1994) designated the species with white body color as "shiroika," which is generally called "aori-ika" in the mainland of Japan (= *S.* cf. *lessoniana* [see section on Taxonomy]); the species exhibiting red body color as "akaika"; and the species with a small body size as described by local fishermen of Ishigakijima Island as "kuaika." Among these three species, "aori-ika" is the most abundant from the mainland of Japan (Honshu) to the Ryukyu Archipelago (Izuka et al. 1996). Previous studies investigating the population genetics of *S.* cf. *lessoniana* have resulted in two mutually exclusive hypotheses. One group used allozyme analysis to examine "aori-ika," a coastal waters species, and reported that the population-level genetic structure differs significantly between the Japan Sea side and the Pacific Ocean side of Japan (Yokogawa and Ueta 2000). Another group analyzed *S.* cf. *lessoniana* from Japan and Thailand and reported that oval squid have a single large gene pool (Izuka et al. 1996, Pratoomchat et al. 2001). Here we address these hypotheses by examining the degree of gene flow and the genetic diversity of *S.* cf. *lessoniana* from East Asia using a noncoding region of mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Taxonomy

Izuka et al. (1994) reported that *Sepioteuthis lessoniana* Férussac, 1831, in Lesson (1830–1831), is heterogeneous; however, this is the extent of its taxonomic classification thus far. This is due in part to the limited number of distinguishing morphological characters but also because the type specimen is no longer available and type locality has not been designated (Lu et al. 1995). This makes it difficult to determine whether genetically recognized species are undescribed species or one of 13 known synonymies (Young 2002). Our ongoing morphological and molecular study may offer a solution. In this study, we refer to the "aori-ika" identified by Izuka et al. (1994) by allozyme analysis as *S.* cf. *lessoniana*.

Sample Collection, DNA Extraction, Amplification, and Sequencing

In total, 242 adult squid were sampled from five localities in Japan and two localities from the East and South China Seas (Taiwan and Vietnam) (Table 1). Japanese samples were collected from five localities in Japanese waters (Ishikawa, Japan Sea; Tokushima, eastern Shikoku; Nagasaki, western Kyushu; Okinawajima and Ishigakijima Island, Ryukyu

TABLE 1

Populations and Samples Used for mtDNA Sequence Analysis in *Sepioteuthis* cf. *lessoniana*

Location	Date	Latitude and Longitude	*n*	No. of Haplotype	*h*	π
Ushitu, Noto Ishikawa Prefecture, Japan	Oct. 2004–Nov. 2005	37° 18′ N, 137° 09′ E	30	4	0.2506 ± 0.1017	0.0007 ± 0.0008
Mugi, Tokushima Prefecture, Japan	July 2004–Nov. 2005	33° 39′ N, 134° 25′ E	30	2	0.0667 ± 0.0613	0.0003 ± 0.0006
Fukue, Goto Islands, Nagasaki Prefecture, Japan	Oct. 2004–Nov. 2005	32° 41′ N, 128° 51′ E	30	5	0.4092 ± 0.1082	0.0059 ± 0.0037
Okinawajima Island, Ryukyu Archipelago, Japan	Apr. 2005–Feb. 2006	26° 35′ N, 127° 58′ E	50	6	0.2939 ± 0.0831	0.0009 ± 0.0010
Ishigakijima Island, Ryukyu Archipelago, Japan	Nov. 2005–July 2006	24° 20′ N, 124° 08′ E	49	6	0.2993 ± 0.0842	0.0039 ± 0.0026
Bisha, Keelung, Taiwan, Republic of China	June 2005–Sept. 2006	25° 09′ N, 121° 55′ E	23	13	0.8972 ± 0.0503	0.0124 ± 0.0070
Gulf of Tonkin, Socialist Republic of Vietnam	Aug. 2004–May 2005	20° 00′ N, 107° 33′ E	30	12	0.6828 ± 0.0958	0.0077 ± 0.0046

Archipelago). We followed the method of allozyme analysis described by Izuka et al. (1994) for species identification. Muscle tissue from an arm was placed in a test tube containing 0.5 ml TNES-8M urea buffer (Asahida et al. 1996). Total DNA was isolated by proteinase K digestion followed by a standard phenol-chloroform extraction. Akasaki et al. (2006) reported that the complete mtDNA of *S. lessoniana* has two noncoding regions that indicated 98% consistency. We analyzed non-coding region 2 (NC2) between Ala and Trp of transfer RNA (tRNA). The original primers, SL-Ala (5′-GGTAACCCTTTCTGTATGATTGC-3′) and SL-Trp (5′-AAAGACCTTGAAAGTC-TTCAG-3′), which target a portion of tRNA-Ala and tRNA-Trp, were used with polymerase chain reaction (PCR) to amplify NC2. PCR reactions were performed in 25 μl using Biotaq (Bioline, Ltd.). A thermal cycler, GeneAmp 9700 (Applied Biosystems), was used with the following parameters: plate-heated at 94°C (120 sec), followed by 30 cycles of denaturation at 94°C (30 sec), annealing at 60°C (30 sec), and extension at 72°C (45 sec). PCR products were purified using a PCR Product Pre-sequencing Kit (USB Co.). The nucleotide sequences were determined using CEQ 8800 (Beckman Coulter) and ABI 3700 (Applied Biosystems) genetic analyzers. All sequences were initially aligned using Clustal-X version 1.83.1 (Thompson et al. 1997), followed by manual editing using MacClade4 version 4.08 (Maddison and Maddison 2005).

Data Analyses

Haplotype diversity (h [Nei 1987]) and nucleotide diversity (π [Tajima 1983]) within populations were calculated using the program Arlequin version 2.000 (Schneider et al. 2000). An analysis of molecular variance (AMOVA [Excoffier et al. 1992]) was used to test population structure within species using Arlequin. Significance thresholds were Bonferroni corrected for multiple pairwise comparisons. Relationships of haplotypes were assessed using a minimum spanning tree created via the Minspanet algorithm in Arlequin and drawn by hand. Dendrograms based on pairwise F_{ST} using Unweighted Pair Group Method with Arithmetic mean (UPGMA [Sokal and Michener 1958]) and neighbor-joining (NJ [Saitou and Nei 1987]) methods were estimated using Phylip version 3.5 (Felsenstein 1991).

RESULTS

A total of 402 base pairs (bp) of NC2 sequences was obtained from 242 *S.* cf. *lessoniana* specimens from seven localities. From a total of 29 haplotypes, 29 variable sites were identified (Table 2). Nine haplotypes were shared between more than two localities, and the remaining 20 haplotypes were specific to a single locality. Among the populations, 86.5% of the samples belonged to haplotype no. 1, which was the major haplotype in all Japanese localities. In contrast, haplotype no. 2 was the major haplotype in Taiwan and Vietnam (Figure 1). Haplotype diversity (h) ranged from 0.0667 in Tokushima to 0.8972 in Taiwan, and nucleotide diversity (π) varied from 0.0003 in Tokushima to 0.0124 in Taiwan (Table 1). The pooled regional genetic variability for Japanese localities was very low ($h = 0.2583$, $\pi = 0.0024$). Conversely, genetic variability values in Taiwan ($h = 0.8972$, $\pi = 0.0124$) and Vietnam ($h = 0.6828$, $\pi = 0.0077$) were higher than that in Japan.

The AMOVA indicated that genetic variation over all localities from Japan was 64.03%, whereas the variation within a locality was 35.97% ($P < 0.01$). The estimated F_{ST} values for the 21 pairs of seven localities ranged from 0 to 0.8593. The 10 pairs formed by combinations of Taiwanese/Vietnamese samples and five localities from Japan had significant F_{ST} values ($P < 0.01$ [Table 3]).

Relationships between haplotypes were represented on a minimum spanning tree, which indicated that the 29 haplotypes were divided into two clusters by eight mutation steps (Figure 2). The two clusters showed obvious haplotype grouping between the Japanese population and the East and South China Seas population. The Japanese cluster

TABLE 2

Variable Sites and Haplotype Distribution of *Sepioteuthis* cf. *lessoniana* among Seven Localities[a]

Haplotype	54	131	160	166	173	214	222	235	237	242	243	244	245	247	252	267	272	274	281	284	285	293	300	307	309	312	334	399	401	IS	TK	NG	OK	IG	TA	VI
1	T	A	A	A	A	T	A	C	T	A	C	C	C	A	T	C	C	A	C	T	A	A	G	A	A	G	A	G	T	26	29	23	42	41	3	2
2	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	·	·	T	·	0	0	3	0	3	7	17
3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	1	0	2	1	1	0	0
4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	0	0	0	0	1	1	1
5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	2	0	0	0	0	1	0
6	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	A	0	1	0	0	0	0	0
7	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	T	T	·	·	·	C	·	·	·	·	·	·	T	·	0	0	0	0	0	1	0
8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	T	·	·	0	0	1	0	0	0	0
9	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0	0	0	3	0	0	0
10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	0	0	0	0	1	0	0
11	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	0	0	0	0	2	0	0
12	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	1	0	0	0	0	0	0
13	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	·	·	·	G	0	0	0	0	0	2	0
14	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	·	C	·	·	·	·	·	·	·	G	0	0	0	0	0	1	0
15	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	·	·	·	·	0	0	0	0	0	1	1
16	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	A	·	T	·	0	0	0	0	0	1	0
17	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	G	C	C	·	·	·	·	·	·	T	·	0	0	0	0	0	2	0
18	·	T	T	·	·	·	G	T	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	·	·	T	·	0	0	0	0	0	1	0
19	·	T	T	·	·	·	·	·	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	·	·	·	·	0	0	0	0	0	0	1
20	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	·	C	·	·	·	·	·	·	·	A	0	0	0	0	0	0	1
21	C	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	·	·	·	·	0	0	0	0	0	0	1
22	T	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	C	C	·	·	C	·	·	·	T	·	0	0	0	0	0	1	2
23	·	T	T	·	·	·	·	T	A	·	A	·	T	G	G	·	T	·	·	C	C	·	·	·	·	·	·	T	·	0	0	0	0	0	1	1
24	·	T	T	·	·	·	·	T	A	·	A	·	T	G	G	·	T	·	·	C	C	·	·	·	G	·	·	T	·	0	0	0	0	0	0	1
25	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	0	0	1	1	0	0	0
26	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	0	0	0	2	0	0	0
27	·	T	T	·	·	·	·	T	A	G	A	·	T	·	G	·	T	·	·	C	C	·	·	·	·	·	·	·	·	0	0	0	0	0	0	1
28	·	T	T	·	·	·	·	T	A	·	A	·	T	·	G	·	T	·	·	·	C	·	·	·	·	A	·	·	·	0	0	0	0	0	0	1
29	·	·	·	·	·	C	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0	0	0	1	0	0	0
Total																														30	30	30	50	49	23	30

[a] IS, Ishikawa; TK, Tokushima; NG, Nagasaki; OK, Okinawajima Island; IG, Ishigakijima Island; TA, Taiwan; VI, Vietnam.

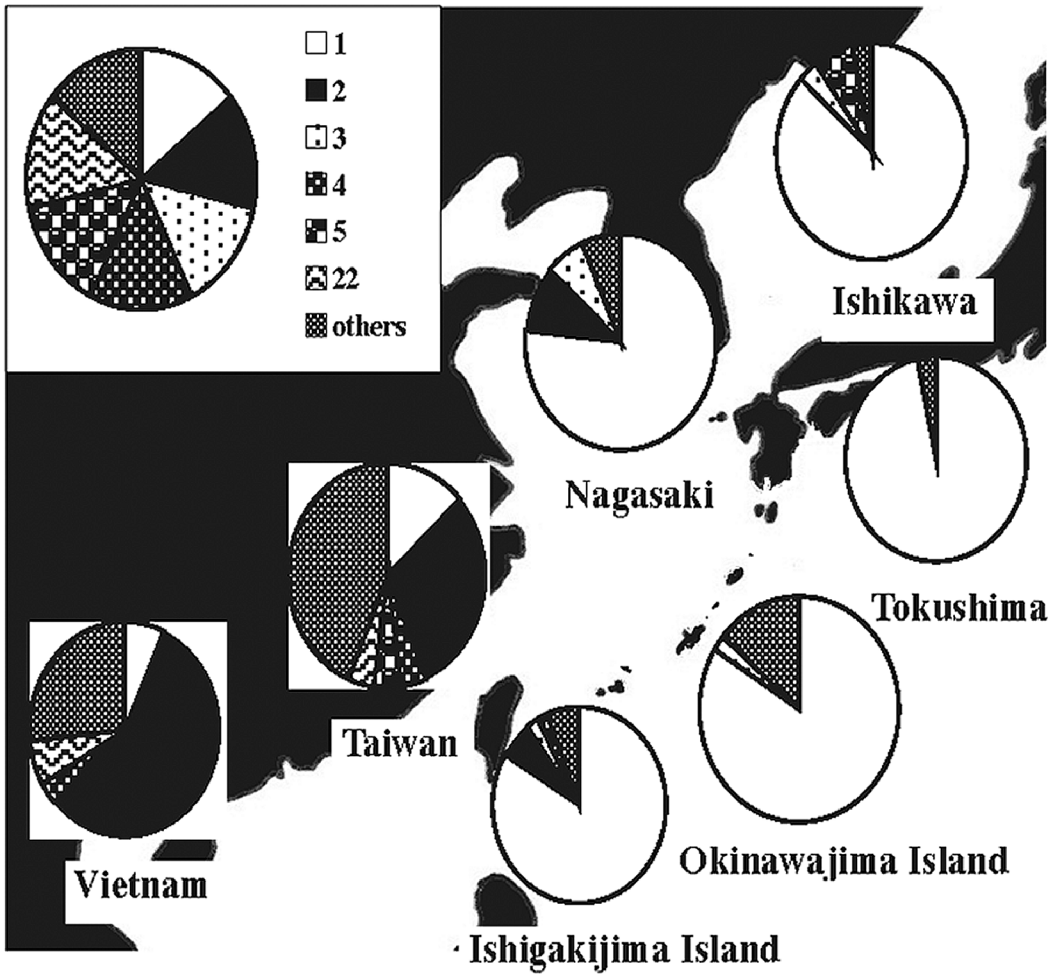

FIGURE 1. Haplotype frequencies of *Sepioteuthis* cf. *lessoniana* from seven localities.

TABLE 3

Pairwise F_{ST} and Associated Probability (P) of *Sepioteuthis* cf. *lessoniana* among Seven Localities

	Ishikawa	Tokushima	Nagasaki	Okinawajima Island	Ishigakijima Island	Taiwan	Vietnam
Ishikawa		0.2252	0.2162	0.1441	0.2793	0.0000*	0.0000*
Tokushima	0.0060		0.1622	0.2703	0.1712	0.0000*	0.0000*
Nagasaki	0.0571	0.0454		0.0270	0.5946	0.0000*	0.0000*
Okinawajima Island	0.0065	0.0112	0.0761		0.0360	0.0000*	0.0000*
Ishigakijima Island	0.0197	0.0113	–0.0151	0.0329		0.0000*	0.0000*
Taiwan	0.7410	0.7312	0.5766	0.7818	0.6711		0.3063
Vietnam	0.8381	0.8308	0.7046	0.8593	0.7713	0.0041	

Note: F_{ST} values are below the diagonal, and corresponding P values are above the diagonal. Bonferroni correction $P^* < 0.05$.

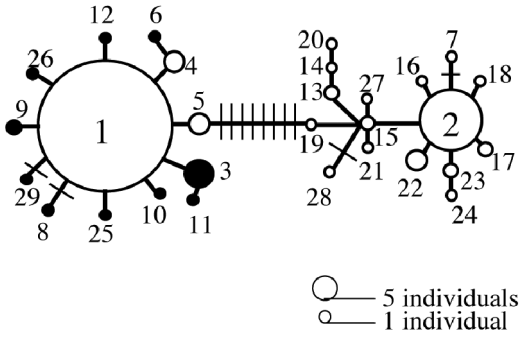

FIGURE 2. Minimum spanning tree among 29 haplotypes of *Sepioteuthis* cf. *lessoniana*. Haplotype no. 1 (*left*), which was more predominant in the Japanese population, is suspected to be the more ancestral type. Black circles indicate unique haplotypes among Japanese localities. The size of the circles is proportional to the haplotype frequencies. All haplotypes are separated by a single substitution step unless otherwise noted by hatch marks or numerals.

appeared starlike, with many individual haplotypes linked by a single or a few mutations, whereas the structure of the East and South China Seas cluster was more complicated. The UPGMA and NJ trees indicated the same topology, and trees constructed with pairwise F_{ST} for the seven populations showed that *S.* cf. *lessoniana* was assigned to two groups (Figure 3). These two groups corresponded exactly with the two clusters predicted by the minimum spanning tree.

DISCUSSION

Marine organisms generally show low levels of genetic differentiation among geographic regions due to high dispersal potential during larval or adult history stages (Palumbi 1994, Grant and Bowen 1998, Hewitt 2000). It is interesting that our results showed significant differences in the genetic structure of *S. lessoniana* from Japan to Vietnam with the AMOVA. Our results differ from those reported by Izuka et al. (1996), and Pratoomchat et al. (2001), based on allozyme analysis, suggested that *S.* cf. *lessoniana* from Japanese waters and Thailand could share a common gene pool over their 2,000 km geographical range. It has been proposed that *S.* cf. *lessoniana* mtDNA is a more sensitive marker than allozyme analysis. On the other hand, Yokogawa and Ueta (2000) reported different genetic structures in Japanese waters. Our results did not show obvious genetic differentiation between populations from the Japan Sea and the Pacific Ocean. Levels of variation in mtDNA may not be sufficient to detect differences in the genetic structure of closely related Japanese populations.

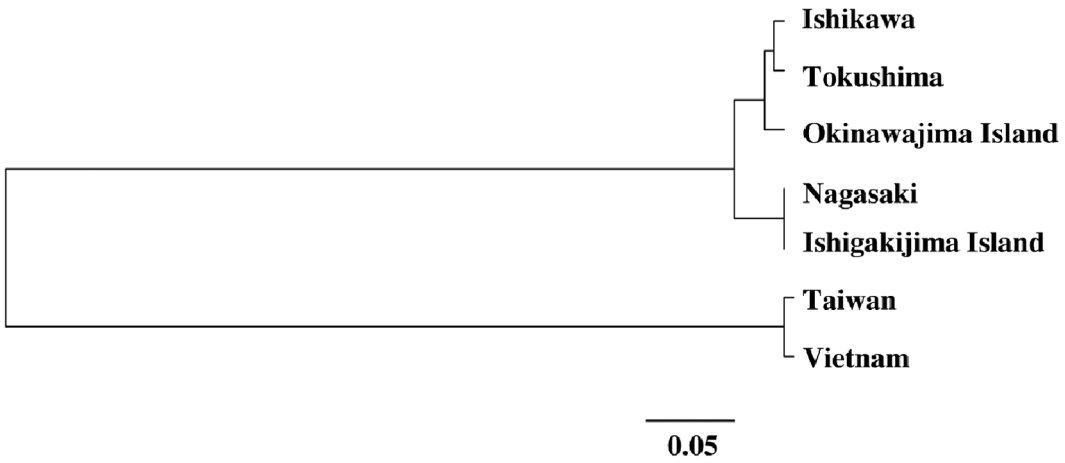

FIGURE 3. Dendrogram constructed by UPGMA based on the pairwise F_{ST} with AMOVA of *Sepioteuthis* cf. *lessoniana*.

Our results indicate that Japanese *S.* cf. *lessoniana* exhibit low genetic variability compared with those of the East and South China Seas ($h = 0.7900$, $\pi = 0.0101$). The average haplotype diversity value for *S.* cf. *lessoniana* ($h = 0.2583$) among Japanese populations was lower than that of the Japanese spear squid, *Loligo bleekeri* Keferstein, 1866 ($h = 0.670$), around Japan with mtDNA noncoding region 4 (Ito et al. 2006). Ito et al. (2006) identified 48 haplotypes from six populations of *L. bleekeri* in Japanese waters, whereas we only identified 13 *S.* cf. *lessoniana* haplotypes from Japanese populations. These results may be explained by some hypotheses. One hypothesis is that *S.* cf. *lessoniana* expanded its distribution into Japanese waters more recently than *L. bleekeri*. The minimum spanning tree also indicated that the expansion of populations in Japanese waters was more recent than that of Taiwanese and Vietnamese populations (Koike 2003). The low genetic variability seen in Japanese populations indicates that *S.* cf. *lessoniana* may have recently colonized Japanese waters, thus suggesting a founder effect or bottleneck. Another hypothesis is that adults of *L. bleekeri* are stronger swimmers than *Sepioteuthis* spp., and the smaller planktonic larvae of *L. bleekeri* may be more easily influenced by a current, although the pelagic larval periods for both species are not known exactly. Therefore, larvae of *L. bleekeri* from abroad may disperse more frequently into Japanese waters. This biological feature may have contributed to the large difference in genetic diversities seen in these Japanese species.

Our results suggest that gene flow between Japanese and East and South China Seas populations has been limited. The boundary between nearby Ishigakijima Island and Taiwan populations is very clear. The geographical distance between Ishigakijima Island and Taiwan is about 300 km, which is the same distance between Okinawajima and Ishigakijima Island. However, we found slightly significant difference in genetic structure in populations between Okinawajima and Ishigakijima Island. These results indicate the presence of barriers that prevent gene flow between Ishigakijima Island and Taiwan. The Kuroshio is a prominent current in this area that moves at a rate close to 50 million m^3/sec (Taira 1997). This may have prevented dispersal from Taiwan to Ishigakijima Island because the main flow of the current passes just north of Ishigakijima Island (Japan Meteorological Agency 2007). In addition, *S.* cf. *lessoniana* tends to prefer coastal waters, which may allow for high genetic diversity in continuous shorelines such as those occurring in Taiwan and Vietnam. These environmental and biological factors may have contributed to the isolated Japanese populations with low genetic variability.

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